



DEVELOPING DRUG THERAPIES:

Cognitive Damage in Mice Following Brain Radiation

Student Author



Rachel Yuska, a senior in health sciences, became involved in research through the Health Sciences Honors Research program. Since her freshman year, she has worked in Dr. Carlos Perez-Torres's lab studying the effects of radiation therapy in

mice. After graduating, Yuska is taking a gap year to work as a medical scribe in Indianapolis before moving on to physician assistant school. She is also a student athlete on the Purdue Cross Country and Track and Field Teams, an ambassador for the College of Health and Human Sciences, and an active member at St. Tom's Catholic Church on campus. In her free time, Yuska likes to hike, hammock at the engineering fountain, and play guitar.

Mentor



Carlos Pérez-Torres is an assistant professor of radiological health sciences within the School of Health Sciences at Purdue University. Dr. Pérez-Torres obtained his BS degree from Worcester Polytechnic Institute in biology and biotechnology

in 2007 and his PhD degree from Baylor College of Medicine in translational biology and molecular medicine in 2012. As a postdoctoral researcher at Washington University in St. Louis, he helped validate and characterize a mouse model of radiation necrosis, a late-onset side effect of radiation therapy that mimics a tumor on standard anatomical MRI. As a faculty member at Purdue University, Dr. Pérez-Torres continues to focus on how radiation treatment affects the normal brain to develop better diagnostic MRI tools (is it a tumor or just a treatment side effect?) and to potentially improve radiation therapy of brain tumors.

Abstract

Pediatric brain cancer patients are at a high risk for radiation-induced cognitive impairment due to white matter changes in the brain. Half of six-month radiotherapy survivors develop significant changes in white matter. Previous research has shown that a mouse model can be used to show similar cognitive and behavioral deficits in human patients. The purpose of this work is to evaluate the effectiveness of two drug therapies, Donepezil and 3,3-Diindolylmethane (DIM), that could be used to either protect the brain from radiation injury or cure the cognitive injury and behavioral deficits that result from whole-brain irradiation. This project consisted of two parts: administration of Donepezil postradiation as a symptomatic cure and administration of DIM before radiation as a protectant. The mice received 30 gray whole brain radiation, and their behavioral changes were measured at 4 and 8 weeks postradiation. Behavioral changes were observed using two tests: the Open Field Test and Marble Burying Test. These tests were to see if the treated mice would have results closer to the healthy baselines established in previous research. From our data, we observed Donepezil to be an ineffective form of therapy, as the deficits did not improve. However, DIM has shown to be a promising protectant drug therapy, as the behavioral data is closer to the results of a healthy control. This research validates the potential of DIM to be used as a radio protectant in preventing both radiation injury and any cognitive deficits from following.

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Keywords

cancer, radiation, mice, brain, behavior, drug therapy, cognitive injury, radiation oncology

GOALS AND AIMS OF THE PROJECT

Over the past four years, my work under Dr. Carlos Perez-Torres has focused on evaluating the neurocognitive effects of radiation on the brain using a mouse model. During my freshman year, our first goal was to identify when late cognitive effects are seen in the brain and understand the extent of damage induced from radiation. We also wanted to find an appropriate radiation dosage that would allow us to model brain damage clearly in a mouse.

We started off by radiating female mice approximately 5–6 weeks old, mimicking a human pediatric brain, with five different radiation dosages of 10, 15, 20, 25, and 30 gray onetime treatment, by use of a linear accelerator. For comparison, the standard therapy dosage for humans ranges from about 14 to 24 Gy for brain metastases (Timmerman, 2008). Then, we monitored the effects of radiation on these brains at different time points postradiation by euthanizing different groups of mice at 1 hour or 4, 8, 12, or 16 weeks. We analyzed the damage to the brain by using immunohistochemistry to stain brain tissue for different kinds of damage. Results showed that 30 Gy was the dosage most effective in showing consistent damage and that damage was the worst at 8 and 16 weeks postradiation.

Next, we investigated the differences between male and female mice in their response to radiation. Initially, the assumption supported by other research showed that female mice were more susceptible to radiation than male mice. So, females would make better models, as their sensitivity would show the effects of injury quite clearly. However, we could not assume that what we were seeing in female mice would be exactly what we might see in male mice. To do this, we radiated both female and male mice with the same dosages of radiation and then used immunohistochemistry to compare the injury between the two sexes. We found that females and males showed radiation injury in different ways. The males showed more injury at a later time point, while the females were more susceptible to the effects of radiation earlier on.

After establishing reliable methods where we could consistently model radiation damage in mice, we wanted to further explore the extent of cognitive damage. The cognitive damage is best observed as behavioral changes. Our primary question was whether mice that received brain radiation developed the same cognitive and behavioral changes as seen in human patients.

In order to track the behavioral changes, we first had to pick behavioral tests to accurately and consistently show these changes. The Open Field and Marble Burying Tests were chosen for their ability to measure normal mouse behaviors, anxiety, and cognitive ability. Both behavioral tests showed a significant cognitive decline in the mice after receiving radiation. The behavioral impairments increased with time, as supported by pathology.

All of this work has led to my final senior project: developing drug therapies for cognitive damage in mice following brain radiation. The purpose of this project was to investigate whether drug therapies, such as Donepezil and 3,3-Diindolylmethane (DIM), can be used in mice to protect from or reverse the cognitive damage that results from brain radiation.

BACKGROUND

Cancer today is considered one of the leading causes of death around the world, especially for children. Brain cancer is among one of those top cancers causing death. For adults, recovery from brain cancer is uncommon because of how aggressive the cancer can be. The survival rate for malignant brain tumors in adults is only 35%, while in children the survival rate is 74% (“Quick Brain Tumor Facts,” n.d.). Radiation is commonly used as an effective form of therapy when treating brain tumors. But like other treatments such as chemotherapy and surgery, radiation comes with risks.

Pediatric brain cancer patients who survive radiation therapy are at a high risk for radiation-induced cognitive impairment in healthy tissue surrounding the tumor due to white matter changes in the brain. Unfortunately, about half of six-month radiotherapy survivors develop significant changes in white matter, potentially leading to significant cognitive changes and impairment. Some of the side effects observed have been learning deficits, a decrease in IQ, or different neurocognitive effects. These side effects are rarely seen in adults, because most adults do not survive long enough after being diagnosed and treated with radiation (Attia et al., 2014). To further investigate these effects, a mouse model of radiation injury was developed to investigate if mice that received brain radiation develop the same cognitive and behavioral changes as seen in human patients.

When radiation doses are used for mice, higher doses must be used, because mice tend to have a higher resistance to the effects of radiation. It is also important to keep in mind that humans and mice

have differences in timing and amount of radiation dosage. Human clinical patients are usually radiated with about 2 Gy, five days a week. However, to mimic that in mice would be extremely expensive and time-consuming. So, mice are usually radiated with a higher dose one time.

Mouse models can be used to show the changes in the brain postradiation, using immunohistochemistry to look for pathological changes and behavior tests to observe changes in behavior. In human patients, behavioral changes include increased anxiety, loss of memory, and a decrease in IQ. Increased anxiety and memory loss are also behaviors that can be measured and observed in mice using the Open Field Test and the Marble Burying Test.

Results showed that brain radiation does cause behavioral changes, and this injury can be modeled in mice to show significant impairments. From a pathology standpoint, radiation of healthy brain tissue resulted in specific changes in white matter consisting of large holes, cell death, and inflammatory responses. This pathology produced deficits that become more pronounced over time, as supported by pathology and behavior tests.

In order to protect from or treat radiation damage of healthy tissue, two drug therapies were chosen to test—Donepezil and DIM. Donepezil, a cognition-enhancing drug used to improve memory and attention in Alzheimer’s patients, is a central acetylcholinesterase inhibitor that is injected intraperitoneally (“Donepezil,” n.d.). It is a drug therapy that focuses on treating the symptoms but cannot cure the underlying disease. Side effects include nausea, vomiting, diarrhea, cramps, weight loss, and dizziness. DIM is a chemical naturally formed in the body by breaking down cruciferous vegetables such as broccoli, cauliflower, and cabbage and is thought to possibly be a protectant from cancer. DIM is a fat-soluble drug that is able to cross the blood-brain barrier, which is an essential characteristic for this therapy (“Diindolylmethane,” n.d.). It is also injected intraperitoneally. Previous literature has proven its use as a protection from radiation damage to skin (Fan, 2013). The only known side effects of the drug are that it can make hormone-sensitive conditions worse, as its mechanism of action is similar to estrogen (“Diindolylmethane,” n.d.).

My project asks whether drug therapies can be used in mice to reverse or protect from the cognitive damage that results from brain radiation. My first

hypothesis is that Donepezil can be used to treat and reverse the effects of radiation injury and cognitive damage, and my second hypothesis is that DIM can be used as a radioprotectant.

MATERIALS AND METHODS

In the previous behavior experiments that did not use a drug therapy, groups of five 4–5-week-old female Balb-c mice were irradiated with 30 Gy whole-brain radiation using an X-RAD 320 (a CabaMed radiator). Before radiation, the mass of each mouse was recorded in order to monitor its health. If the mouse lost too much weight, then it was euthanized. A control group that received no radiation was initially tested to set a baseline for the cognitive abilities and behavior patterns of a healthy mouse. After radiation, mice underwent behavior tests at three time points: 4, 8, and 16 weeks. As previously mentioned, the behavior tests chosen were the Open Field and Marble Burying Tests.

In the Open Field Test, the mouse is placed in an artificially designed field. The field consists of a 40-by-40-inch plastic box with opaque walls. A camera was hung on an apparatus over the top of the field to monitor its movements, as shown in Figure 1a. The mouse was placed in the center of the field and allowed to freely roam for 20 minutes. Controls (mice not treated with radiation) spent an even amount of time on the inside, outside, corners, and middle of the field with a high level of ambulation. Mice that showed cognitive impairments postradiation spent more time on the outside of the field and increased ambulation indicating higher anxiety. Video analysis software with a Matlab code was used to analyze the data.

In the Marble Burying Test, 15 black nonshiny marbles were placed in a 3-by-5 grid in a mouse cage with extra bedding, as shown in Figure 1b. Mice were placed in the center of the cage and allowed to bury and move freely for 20 minutes. At the end of the 20 minutes, the number of marbles buried was counted. If a marble was at least 50% covered by the bedding, it was counted as buried. Control groups were used to set a baseline range of how many marbles a healthy mouse buried (11 marbles) to use as comparison. Mice postradiation with cognitive impairments buried significantly fewer marbles.

For this project the same procedure was followed exactly, only with the addition of drug therapies. In the first experiment Donepezil was used. Starting right after radiation, 5 mg/ml or 50 mg/kg of body weight of Donepezil was administered five times

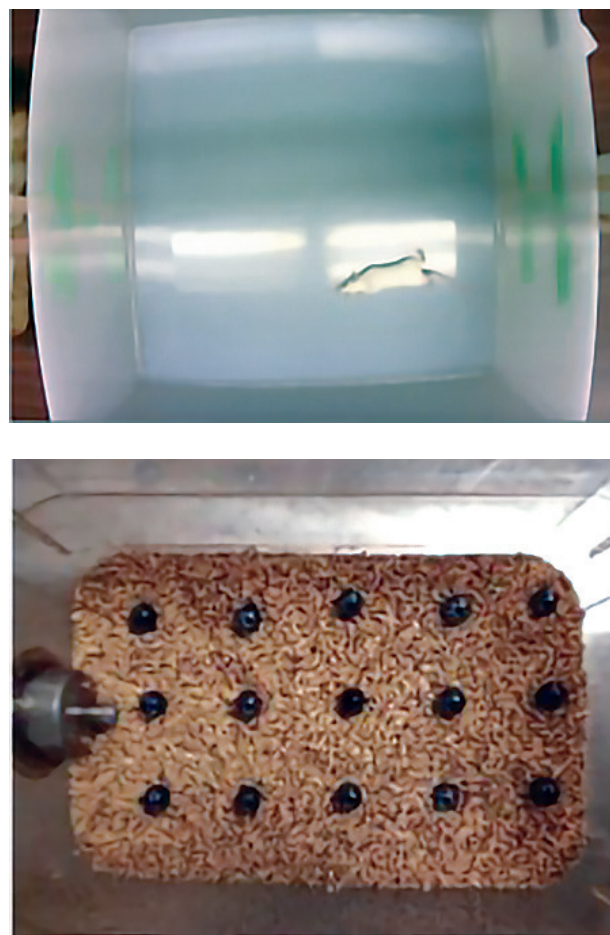


Figure 1. (A) Open Field Test apparatus. A mouse is dropped into the center of the field and allowed to move freely for 20 minutes. Movements of the mouse are tracked with an overhanging camera and analyzed with a Matlab software. (B) Marble Burying Test apparatus. A mouse is dropped into a cage with 15 marbles (3 rows of 5). Each mouse is given 20 minutes to bury the marbles. Normal behavior of a healthy mouse is to bury around 11 marbles.

a week intraperitoneally to try to treat the radiation damage. After radiation and during Donepezil treatments, at 4 weeks and 8 weeks postradiation the mouse underwent both the Open Field Test and the Marble Burying Test. In the second experiment, 5 mg/ml of DIM was administered 30–40 minutes before radiation. The allotted time was to allow the drug to spread throughout the body and cross the blood-brain barrier. The mice were also tested at 4 and 8 weeks postradiation with the behavior tests. The sample size for the controls was 13, mice treated with Donepezil was 5, and mice treated with DIM was 7.

Qualitative observations were taken throughout the entirety of the experiments, and quantitative data was recorded and analyzed by a one-way ANOVA

Dunnett and Bonferroni statistical analysis. Averages of thigmotaxis and ambulation were compared, as were the medians of the number of marbles buried. The significant p-value chosen for difference between groups was 0.05.

RESULTS

From the Matlab code analyzing the video of the Open Field Test, a heat map was produced in one image and a line trace of all of the movements of the mouse during the 20 minutes in the open field in another image. For the images produced

following the line of movement for the mouse, the image contains two red boxes: one to differentiate what was considered the inner quadrant and the other to differentiate what was considered the outer quadrant. This distinction was important in analyzing thigmotaxis, a ratio of the time the mouse spent on the outside divided by the total time. Thigmotaxis is a useful measure of anxiety, as an increased thigmotaxis is associated with increased anxiety in those mice with cognitive impairments. Figures 2a–c show the movement tracings of the mice that received only radiation and no treatment, and below that in Figures 2d–g are the movement

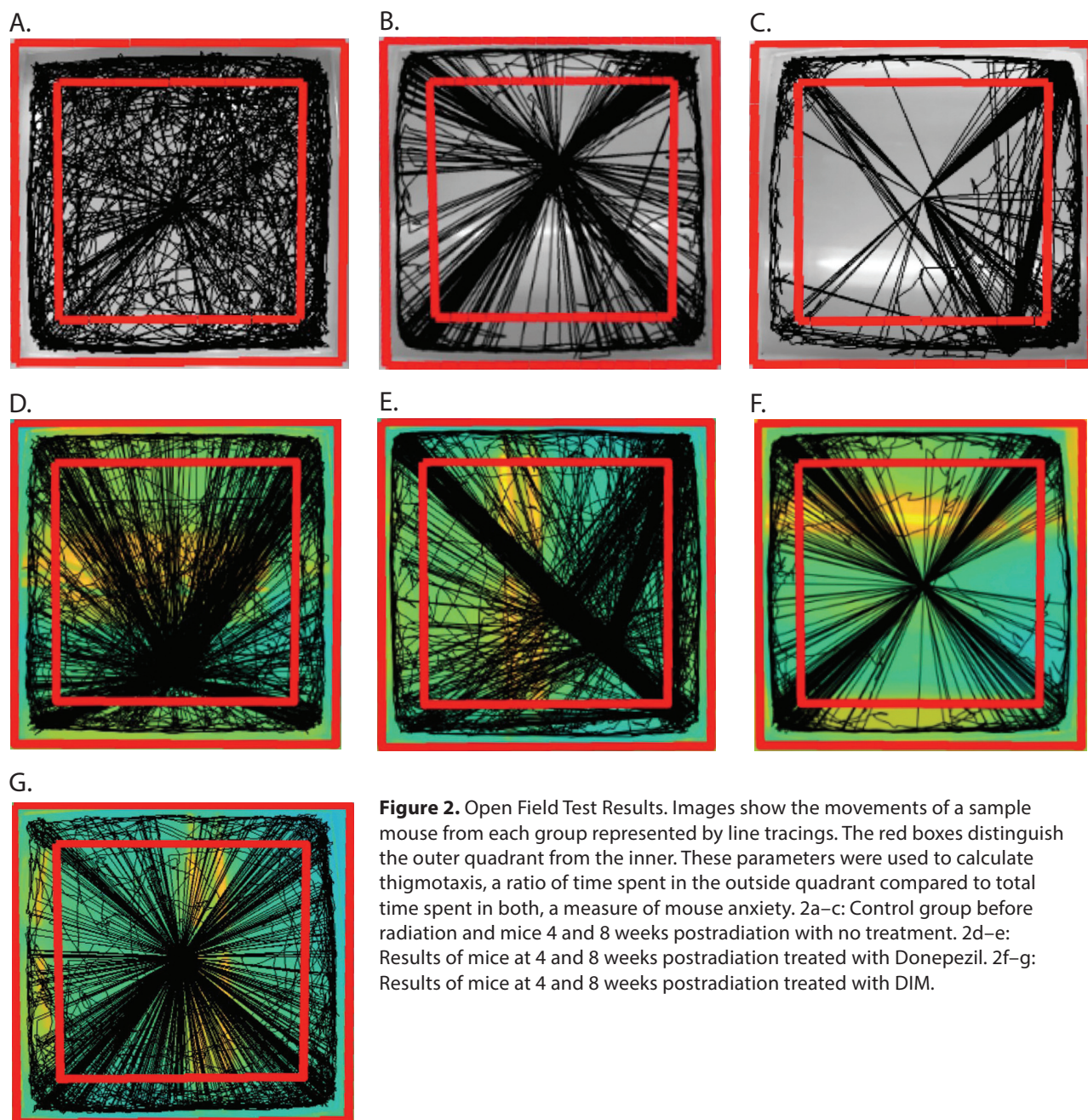


Figure 2. Open Field Test Results. Images show the movements of a sample mouse from each group represented by line tracings. The red boxes distinguish the outer quadrant from the inner. These parameters were used to calculate thigmotaxis, a ratio of time spent in the outside quadrant compared to total time spent in both, a measure of mouse anxiety. 2a–c: Control group before radiation and mice 4 and 8 weeks post-radiation with no treatment. 2d–e: Results of mice at 4 and 8 weeks post-radiation treated with Donepezil. 2f–g: Results of mice at 4 and 8 weeks post-radiation treated with DIM.

tracings of the mice that received Donepezil and DIM treatments.

Based on the qualitative observations of these movement tracings, the pictures of the mice treated with Donepezil looked very similar to those of the mice at 8 weeks that did not receive any treatment. There are many lines pointing to the corners, indicating that the mice spent more time on the outside. At 4 weeks postradiation, the DIM pictures looked similar to the 4 weeks with no treatment pictures. However, the 8-week postradiation picture looked more similar to the control that received no radiation, as the movement appears to spread evenly throughout the field.

To quantitatively analyze the Open Field Test data, a statistical analysis was performed on the data generated by the video Matlab code. Two important measures were used to observe the differences between the controls and the treated mice: average total ambulation and average thigmotaxis. Figures 3 and 4 show the results of the Open Field Test quantitative analysis.

In Figure 3, all the groups were statistically significantly different from the control. Ideally, the treatment groups would not be different from the control, as the control is the baseline for a healthy mouse. For average total ambulation, at 4 weeks the treatments did not look different from the group that did not receive a drug therapy. At 8 weeks, the same amount of average ambulation was maintained for both drug therapies. Donepezil appeared to be getting closer to the control as time went on but was not close enough to be statistically significant. In Figure 4, the average thigmotaxis for both DIM and Donepezil was not significantly different from the control, indicating that anxiety might have been decreased in the mice. However, by 8 weeks only the DIM therapy maintained a statistically similar thigmotaxis. So, it is possible that DIM, based on this graph, could potentially have protected against the radiation damage. Donepezil might have treated a little bit of the damage, as observed in the average total ambulation and average thigmotaxis, but the therapy was not as effective as the deficits became more pronounced. It was noticed that total average ambulation might not be an accurate measure of cognitive health, as some mice showed anxiety through hyperactivity of increased movement, while other mice showed anxiety through little or no movement.

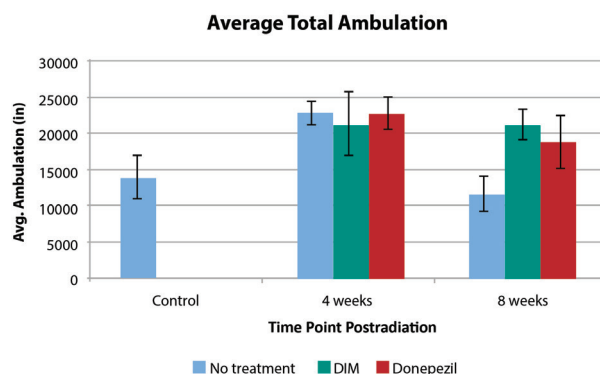


Figure 3. Graph of average total ambulation. Data is presented as mean with standard deviation. Blue bars show the control group as a comparison ($n = 10$) and the average ambulation of mice who did not receive any treatment at 4 weeks postradiation and 8 weeks postradiation ($n = 7$). The green bar shows mice that received DIM before radiation at 4 and 8 weeks postradiation ($n = 7$), and the red bars show mice that received Donepezil treatments after radiation ($n = 5$). ANOVA statistical analysis showed each group to be statistically different from the control group.

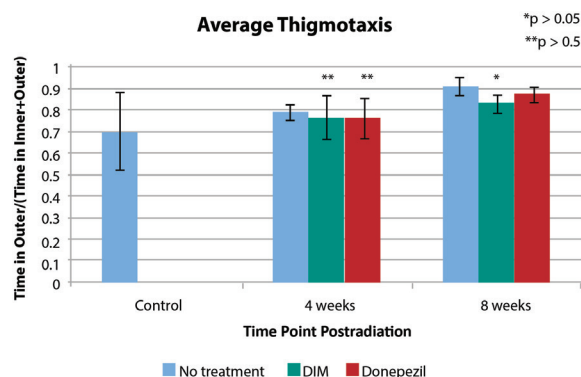


Figure 4. Graph of average thigmotaxis. Data is presented as mean with standard deviation. Thigmotaxis is a measure of mouse anxiety and is calculated by dividing the total time the mouse spent on the outer quadrant by the time spent in both the outer and inner quadrants. ANOVA statistical analysis showed that both groups at 4 weeks postradiation treated with Donepezil or DIM were very statistically similar to the control ($p > 0.5$). At 8 weeks postradiation, only the group treated with DIM remained statistically similar to the control ($p > 0.05$).

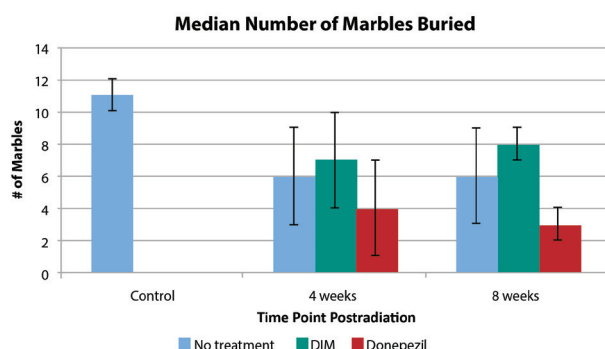


Figure 5. Graph of median number of marbles buried. Data is presented as median with standard median absolute deviation to reduce the effect of outliers. Mice treated with DIM consistently buried more marbles than the group that did not receive any drug therapy and the group treated with Donepezil and was closest to the control median.

For the Marble Burying Test a significant number of outliers were observed, as some groups were consistent, while others contained many individuals who varied greatly in the number of marbles they buried. Therefore, the median was chosen as the appropriate means of comparison between the groups. Figure 5 shows the quantitative results of the Marble Burying Test. The median absolute deviation was used to draw error bars.

Figure 5 shows that DIM was the only effective therapy that actually showed improvements from the group that received no treatment. The use of Donepezil actually worsened the effects of the cognitive damage, as mice treated with this drug performed much more poorly on the test. From a qualitative perspective, it was noted that the mice treated with DIM seemed to be much more mobile, active, and healthy than the mice who did not receive treatment. Those mice that were treated with Donepezil were less mobile and active overall and seemed sicker. Their overall manner was more similar to the mice with significant cognitive damage that received no treatment and did not bury very many marbles.

DISCUSSION OF THE RESULTS

After collecting and analyzing all of the data from the Open Field Test and the Marble Burying Test and comparing it to the control groups that received no treatment, it was apparent that individual variation played a large role in the data. A larger sample size could decrease this variation. Despite the noisy data, the results shown did increase knowledge about what kind of drug might work best to protect from or treat the brain following radiation damage.

The Open Field Test showed that Donepezil might have been a little effective at reducing anxiety initially but was not strong enough to fight the worsening cognitive impairment. DIM proved more promising long-term, as it was able to produce a thigmotaxis similar to the control, implying less anxiety.

The Marble Burying Test showed that DIM was much better at lessening the cognitive impairment of the radiation damage in the mice. It is important to note that it did not protect the mice completely from the damage but did possibly slow down its onset or lessen the impairment. Donepezil did not show improvements in cognitive ability.

CONCLUSION

Donepezil proved to be an ineffective form of correction therapy, as it did not lessen the extent of radiation damage to an effective degree. In the Marble Burying Test, mice treated with Donepezil buried a much smaller proportion of the marbles than the control mice or even those mice who did not receive any treatment. Donepezil might have treated some damage initially as observed in the Open Field Test, but the therapy was not effective as time went on. However, both the Open Field Test and the Marble Burying Test showed DIM to potentially be an effective form of protective radiation therapy for pediatric cancer patients who are at risk for white-matter injury. In the Marble Burying Test, mice treated with DIM buried more marbles than the mice that received no treatment. The most promising indicator of DIM's potential or of drug therapies whose mechanism is similar to DIM's was the qualitative observations. If DIM was able to improve the overall health for most mice, then it might be protecting from more radiation damage and cognitive impairment than it shows quantitatively in the behavior tests.

FUTURE DIRECTIONS

In the future, this experiment could be greatly improved with an increase in sample size. This would hopefully reduce the individual variability of the results. The lab is also planning on continuing to use behavior tests to further study the effectiveness of DIM and possibly other drugs with a similar radioprotectant mechanisms. The ultimate purpose of the research is to safely and effectively treat humans for and protect them from the damaging effects of radiation. In the next step toward clinical use, a mouse tumor model must be developed to investigate if DIM protects tumor tissue as well as healthy tissue. There are a few new questions that have come about from this research: Are there better behavioral tests that can show the cognitive impairment as seen in human patients with radiation injury? By what exact mechanism does DIM work? What is the most important factor of a radioprotectant? Does DIM improve the pathological injury also? Can we see those changes through immunohistochemistry? Investigating the answers to these questions is the next important step in furthering this research.

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